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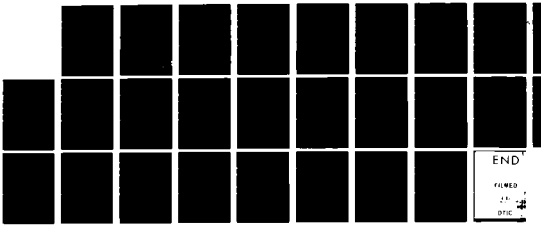

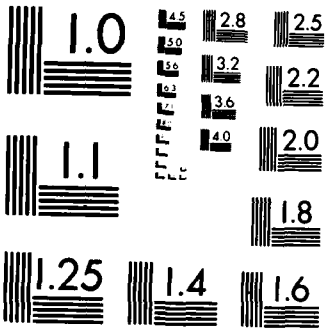

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OVIPOSITION-MODIFYING SUBSTANCES FOR MOSQUITOES

Annual Summary Report

Yih-Shen Hwang

September 1, 1979

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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Department of Entomology, University of California
Riverside, CA 92521

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

To prepare for the field evaluation of the previously identified oviposition repellents, their concentration-activity relationship and species specificity were studied. The magnitude of repellency of the repellents was found to be directly proportional to their concentrations. Butyric acid, the major repellent component, was repellent to Cx. p. quinquefasciatus, Cx. tarsalis, Ae. aegypti, and An. quadrimaculatus at various concentrations. The acid was repellent to Cs. incidens at higner concentrations but attractive at lower concentrations.

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Our sensory physiology studies showed that the chemoreceptors for the perception of butyric acid were located in the antennae which might be the most important sensory organ in mediating the negative ovipositional responses of the mosquitoes to the repellents.

The oviposition attractants produced by microbial fermentation of a chicken manure infusion were found to be distillable with steam and extractable with organic solvents. Isolation and identification of the oviposition attractants are currently under way.

SUMMARY

The objectives of this research project are to investigate the chemistry and biology of oviposition-modifying substances for various species of mosquitoes, to study the possibility of applying these substances in the management of mosquito populations, and to evaluate the role of oviposition attractants for sampling female adults and their ovipositional activity. We previously accomplished the isolation and identification of mosquito oviposition repellents and biological characterization of these oviposition-modifying substances. In the initial stages of the research program, our purposes are to complete preparatory work for the imminent semi-field and field evaluation of mosquito oviposition repellents, to study the sensory physiology of oviposition repellents, and to develop procedures and techniques for the isolation and identification of oviposition attractants.

To determine the activity of the previously identified oviposition repellents quantitatively and to study the relationship between their concentrations and biological activity, six repellent carboxylic acids were bioassayed against Culex pipiens quinquefasciatus Say and Cx. tarsalis Coquillett at various concentrations in olfactometer units. The most effective repellents for Cx. P. quinquefasciatus were acetic and isobutyric acids, and that for Cx. tarsalis was caproic acid. Within the range of concentrations used, the magnitude of repellency of the carboxylic acids was directly proportional to the acid concentrations.

To determine the species specificity of the repellents, butyric acid, the major repellent component, was bioassayed against several species of mosquitoes other than Culex at various concentrations. Aedes aegypti L. females showed significant negative response to butyric acid at $6x10^{-1}$% concentration. Anopheles quadrimaculatus Say was significantly repelled by the acid at $6x10^{-2}$ and $6x10^{-3}$%. Culiseta incidens (Thomson) females were significantly repelled by butyric acid at $6x10^{-1}$%, but no significant response was observed at $6x10^{-2}$%. Nonetheless, at both $6x10^{-3}$ and $6x10^{-4}$%, Cs. incidens females were significantly attracted by butyric acid. Noteworthily, butyric acid showed remarkable properties of being both attractant and repellent for this species of mosquito dependent upon its concentrations.

For investigating the structure-activity relationship and for attempting to find more effective repellents, some homologues of the previously identified carboxylic acids were bioassayed for their ovipositional activity. Caprylic acid showed 100% repellency at $6x10^{-3}$ and $6x10^{-4}$% concentrations against Cx. p. quinquefasciatus. Caproic acid was significantly repellent at $6x10^{-5}$%. Lauric acid was significantly repellent at $6x10^{-4}$ and $6x10^{-5}$%.

In conducting field tests, it is very difficult to assess the effectiveness of oviposition repellents which may possess larvicidal activity. It was therefore important to evaluate these oviposition repellents for their larvicidal activity. Our preliminary studies revealed that caprylic, capric, and lauric acids did not exert any larvicidal activity against second instars of Cx. p. quinquefasciatus at 1, 5, and 100 ppm concentrations.

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To identify the locations of chemoreceptors which mediate negative ovipositional responses of mosquitoes, the proboscis, all tarsal segments, and a variable number of antennal segments of Cx. p. quinquefasciatus females were extirpated, and the ovipositional responses of the extirpated mosquitoes were studied quantitatively using butyric acid as a repellent. The location of the chemoreceptors for perceiving butyric acid was thus determined to be in the antennae which could be the most important sensory organ to mediate the negative ovipositional responses.

Concurrent with the oviposition repellent studies, techniques and procedures are being developed for the isolation and identification of oviposition attractants produced by microbial fermentation of chicken manure infusions. Steam distillation of the active chicken manure infusions produced an active distillate which, upon ether extraction, yielded an active ether extract. Further purification of the attractive fraction is presently under way.

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ANNUAL REPORT

A. INTRODUCTION

Gravid females of many species of mosquitoes show a high degree of preference in selecting specific oviposition sites in the general area of their breeding sources. This preference may be due to the presence of oviposition pheromones or oviposition attractants and repellents in natural habitats. These oviposition-modifying chemicals regulate the ovipositional behavior of mosquitoes and thus provide a mechanism for the prevalence and occurrence of adult mosquitoes. Oviposition pheromones may occur in nature as intraspecific messengers to inform conspecifics of suitable oviposition sites. Oviposition attractants and repellents are generally believed to be produced in nature by microbial fermentation and breakdown of organic matter, and these transspecific products serve as kairomones or allomones providing cues for gravid mosquitoes to detect suitable or unsuitable oviposition sites. If these oviposition-modifying substances become known and available to us, mosquito populations can be sampled and manipulated through regulation of mosquito oviposition. Thus, these substances offer good potential as mosquito-control agents which could supplement other chemical and biological control strategies developed for vector and pest mosquitoes. Additionally, a knowledge of these behavior-modifying substances could provide a basis for understanding behavioral responses of gravid females which constitute an important portion of the total populations.

B. BACKGROUND

The presence of attractants and repellents in natural habitats for mosquito oviposition has been demonstrated by many researchers. Gerhardt (1959) reported that ovipositing female mosquitoes were attracted by stimuli, acting on their olfactory receptors and directing them to oviposition sites containing excess organic matter. In qualitative studies, Gubler (1971) showed that ovipositing females of Aedes (Stegomyia) albopictus Marks and Ae. (S.) polynesiensis Marks were repelled by high concentrations of ammonia and protein solutions, but attracted by high concentrations of organic infusions such as leaf, grass, and guinea pig chow. Gjullin et al. (1965) reported that grass infusions and log pond water increased oviposition of Ae. aegypti L. and Culex pipiens quinquefasciatus Say. Ikeshoji (1966a, 1968) showed the existence of oviposition attractants for Cx. p. fatigans Wiedemann in natural breeding water and attempted to isolate and identify them. Crude mixtures of oviposition attractants for Cx. p. quinquefasciatus, Cx. tarsalis Coquillett, Ae. nigromaculis (Ludlow), and Ae. taeniorhynchus (Wiedemann) were isolated from natural breeding water by Ikeshoji and Mulla (1970). Several workers produced evidence showing that bacteria produced certain oviposition attractants and stimulants as degradation products of organic matter (Hazard et al. 1967, Maw 1970, Ikeshoji et al. 1975).

From the evidence in the literature and from our own preliminary investigations, it is evident that, in natural breeding sites, microbial decomposition of certain organic matter produces volatile and non-volatile substances which act as oviposition-modifying factors for various mosquitoes. These substances may be species-specific or non-specific. They can be attractants, repellents,

stimulants, or deterents.

In view of these successful approaches to produce mosquito oviposition-modifying substances in infusions of organic matter, we initiated exploratory studies on the biology and chemistry of some fermentation products of organic matter showing oviposition-modifying activities. After investigating infusions of various organic substrates, we found that a 1% lab chow infusion was significantly repellent to several species of ovipositing female mosquitoes and that a 1% chicken manure infusion was significantly attractive to Cx. p. quinquefasciatus females but repellent to Cx. tarsalis females (Kramer and Mulla 1979a). These infusions were prepared by adding 1 part of lab chow or chicken manure in 100 parts of tap water and allowing the resulting suspensions to ferment at room temperature. The lab chow infusion became repellent after five days and stayed active for two more weeks. The chicken manure infusion showed attractancy from 9 to 26 days after the start of fermentation.

For carrying out a number of different chemical and biological investigations, a large amount of stock infusions was needed. It thus became necessary for us to determine the storage stability of the infusions at various temperatures. Our studies showed that both infusions remained active for ten weeks or longer when stored in a freezer at -10°C. This storage technique has enabled us to store a large quantity of active infusions for considerable period of time. The storage stability was short-lived at 10° or 20°C.

To characterize the response of mosquitoes to the oviposition repellents, the effects of several physiological parameters on the responses of mosquitoes were investigated. We found that the interval between blood feeding and oviposition, the parous condition, and prior exposure to test infusions in bioassays did not affect the response of gravid mosquitoes to the 1% lab chow infusion.

As previously described, the infusions were not active when freshly prepared, but they gradually became active as the aging process proceeded. It was suspected that bioactive compounds were produced as a result of microbial fermentation. The involvement of microorganisms in the production of attractants and repellents was determined by comparing the activity of the infusions prepared and brewed under septic and aseptic conditions. The aseptic infusions remained inactive whereas the septic infusions consistantly showed repellency or attractancy (Kramer and Mulla 1979a).

The production of active compounds by microorganisms in organic infusions was thus confirmed, and the biological activity of these compounds was characterized. Consequently, chemical investigations seemed to be in order for the determination and structural elucidation of the active compounds which elicited positive or negative oviposition response in mosquitoes. In our studies on the isolation and chemical identification of the oviposition repellents, the active lab chow infusion was distilled to give an active distillate which, upon ether extraction, gave an active ether extract. Fractionation of the ether extract yielded an active acidic fraction and an inactive non-acidic fraction. Gas chromatographic analysis on Porapak R and AT-1200--H_3PO_4 columns of the acidic fraction showed the presence of acetic, propionic, isobutyric, butyric, isovaleric, and caproic acids, butyric acid constituting 85% of the total amount of these acids. In bioassay tests, these aliphatic carboxylic acids, indivi-

dually and in combination, exhibited ovipositional repellency against the two species of Culex mosquitoes at the concentration of $6x10^{-2}$% (Hwang et al. 1978, 1979).

C. OBJECTIVES

The objectives of this research project are:

1. To investigate the chemistry of oviposition-modifying substances for various species of mosquitoes.

The oviposition-modifying substances for mosquitoes will be isolated and purified from natural sources, and active compounds will be chemically identified or their structures will be elucidated. If the active compounds are novel and commercially unavailable, they will be synthesized. Analogues or homologues of the active compounds will also be synthesized for structure-activity studies.

2. To investigate the biology of oviposition-modifying substances.

Responses of various species of ovipositing female mosquitoes to the oviposition-modifying substances will be studied in the laboratory. The sensory physiology and the toxicity of these behavior-modifying substances will be investigated. Concentration-activity relationship and species specificity of these substances against various species of mosquitoes will be studied.

3. To study the possibility of applying oviposition-modifying substances in the management of mosquito populations.

To achieve this goal, these substances will be evaluated under semi-field and field conditions against various species of mosquitoes. This will allow us to assess the effectiveness of using the oviposition-modifying substances to manipulate ovipositional behavior of mosquitoes under field conditions.

4. To evaluate the role of oviposition attractants for sampling populations of female adult mosquitoes and ovipopulations.

The oviposition attractants will be evaluated in traps or light traps to assess their efficacy for collecting female adult mosquitoes and eggs for population and epidemiological studies. By doing so, mosquitoes that do not readily respond to conventional light traps may possibly be collected.

D. SPECIFIC AIMS

The specific aims of the present research program are:

1. To isolate and purify oviposition-modifying substances from infusions of various organic materials that possess oviposition-modifying activity by means of physical and chemical procedures in pure or semi-pure forms for chemical identification.

2. To chemically identify or to elucidate the structures of the oviposition-modifying substances with various spectrometric and chromatographic techniques.

3. To synthesize the oviposition-modifying substances, if they are novel and commercially unavailable, by means of modern methods of organic synthesis.

4. To synthesize homologues and analogues of oviposition-modifying substances for procuring more active compounds and for structure-activity relationship studies.

Physical properties and structural characteristics of these compounds will be used as parameters to correlate their chemical structures with ovipositional activity against several important species of pest and vector mosquitoes.

5. To study ovipositional responses of gravid female mosquitoes to the oviposition-modifying substances in laboratory olfactometers.

6. To investigate the sensory physiology of the oviposition-modifying substances and to identify the locations of chemoreceptors that mediate the perception of the oviposition-modifying substances in mosquitoes.

7. To evaluate the possible toxicity of the oviposition-modifying substances--the toxicity which may possibly influence the accuracy of field evaluations of these behavior-modifying substances.

8. To study the concentration-activity relationship and the species-specificity of the oviposition-modifying substances against such mosquitoes as Culex, Aedes, and Anopheles.

9. To evaluate the oviposition-modifying substances under semi-field conditions using galvanized sheet-metal cylinders in natural mosquito-breeding ponds.

10. To evaluate the oviposition-modifying substances under field conditions using natural mosquito-breeding ponds to assess the effectiveness of these substances in manipulating ovipositional behavior of various species of mosquitoes in the field.

11. To evaluate the feasibility of utilizing the oviposition attractants in traps or light traps for sampling populations of mosquitoes that do not readily respond to conventional mosquito light traps.

F. CURRENT ACCOMPLISHMENTS (APRIL-AUGUST 1979)

E-1. CONCENTRATION-ACTIVITY RELATIONSHIP OF OVIPOSITION REPELLENTS

Our previous studies showed that acetic, propionic, isobutyric, butyric, isovaleric, and caproic acids, individually and in combination, exhibited ovipositional repellency against Cx. p. quinquefasciatus and Cx. tarsalis at

the concentration of $6x10^{-2}$% in laboratory olfactometer units (Hwang et al. 1978, 1979). To determine the biological activity of these carboxylic acids quantitatively and to investigate the relationship between their concentrations and biological activity, the present studies were conducted (Kramer et al. 1979).

Methods and Materials. The mosquitoes used in this study were obtained from stock colonies maintained in our laboratory. Procedures used for the maintenance of laboratory colonies were described by Kramer and Mulla (1979a).

To conduct bioassay tests, two glass stender dishes (37x25 mm with a 4.735 cm^2 surface area) were placed on a paper towel, over which a 1-liter polystyrene cup (Amoco #41032) was inverted. An aqueous solution or suspension of a test compound (4 ml) was placed in one of the dishes, and a distilled water standard (4 ml) was placed in the other. A vacuum line (flow rate, 1 liter/min) was connected to the top of the bioassay unit to provide for ventilation and to create a gradient of the volatile test compound. Five females (6-8 days post bloodfeeding on chicks) were introduced into the bioassay unit between noon and 3 p.m. on the test date, and the results of oviposition were recorded the following morning. All experiments were replicated at least four times.

The criterion for the measurement of oviposition response was the number of ovipositions in both the treatment and the standard. The activity is expressed as the oviposition activity index (OAI) and calculated as follows:

$$OAI=\frac{N_t - N_s}{N_t + N_s}$$

wherein N_t denotes the number of ovipositions in a treated sample, and N_s denotes the number of ovipositions in the standard (untreated). In Culex mosquitoes, the number of ovipositions was determined from the number of egg rafts observed.

All index values determined by this formula lie within the range from +1 to -1. Positive values indicate that more ovipositions are observed in the treatment than the standard, thus evincing the compound to be attractive. Negative values indicate that more ovipositions are observed in the standard than the treatment, thus showing the compound to be repellent. The data were analyzed statistically and the significance of all indices was determined by the chi-square analysis.

All aliphatic carboxylic acids used were of reagent grade. Except caproic acid, they were dissolved in distilled water and serially diluted to the desired concentrations. Caproic acid was dissolved in acetone, and the acetone solution was serially diluted. Aliquats of the serially diluted acetone solutions were dispensed in water to make aqueous solutions or suspensions at the desired concentrations. No more than 1% of acetone was present in the resultant solutions or suspensions. In testing caproic acid in various concentrations, an equal amount of acetone was added to the standard dish.

To assess synergistic action of the six carboxylic acids, they were mixed in combination at both the original ratio found in the 1% lab chow infusion (combination 1: 87.58% butyric acid, 3.79% acetic acid, 3.40% caproic acid, 3.12% isovaleric acid, 1.43% isobutyric acid, and 0.08% propionic acid) and at an equal ratio (combination 2).

Results and Discussions. Figure 1 shows the ovipositional response of Cx. p. quinquefasciatus and Cx. tarsalis females to the six lower aliphatic acids at the concentrations from $6x10^{-1}$ to $6x10^{-4}$%. All six acids were significantly repellent to both species of mosquitoes at $6x10^{-1}$% and to Cx. p. quinquefasciatus at $6x10^{-2}$%. Acetic and isobutyric acids showed significant repellency against Cx. p. quinquefasciatus at $6x10^{-3}$%. Significant negative responses were displayed by Cx. tarsalis to caproic acid at $6x10^{-2}$ and $6x10^{-3}$%. At lower concentrations, these acids induced no significant responses in the mosquitoes. The concentration of the mixture of these acids in the original lab chow infusion was $6x10^{-2}$%.

Our studies revealed that the most effective repellents for Cx. p. quinquefasciatus were acetic and isobutyric acids and that for Cx. tarsalis was caproic acid. Within the range of concentrations used, the magnitude of repellency of the carboxylic acids was directly proportional to the acid concentrations.

To determine if there was any significant relationship between the acid concentrations used and the resulting numbers of oviposition observed, the mean total number of ovipositions (treatment and standard) for each unit in the oviposition choice tests was computed for all acids tested against both species of mosquitoes and subjected to the analysis of variance. Significant F values were determined for butyric and acetic acids against Cx. p. quinquefasciatus and for isobutyric acid against Cx. tarsalis. Means were ranked by using Duncan's new multiple range test, and the results are shown in Table 1. These studies revealed that there was a significant inverse relationship between the acid concentrations and the mean number of total ovipositions in a unit.

Table 2 shows the ovipositional activity of combinations 1 and 2 against Culex mosquitoes. The responses of mosquitoes to both combinations were very similar at the $6x10^{-2}$ and $6x10^{-1}$% concentrations. Neither combination induced a response greater than that produced by the individual acids at the corresponding concentrations. From these investigations, it became apparent that each carboxylic acid acted individually as oviposition repellent and no synergistic effect was found by combining these acids altogether.

E-2. SPECIES SPECIFICITY OF OVIPOSITION REPELLENTS

Because some species of mosquitoes cohabit the same aquatic environment, they might utilize the same chemical substances for selecting oviposition sites. Prior to field experiments, it was therefore essential to determine the ovipositional activity of the repellents against several species of mosquitoes other than Culex for understanding the species specificity of the repellents.

Methods and Materials. Butyric acid was found to be the major component in the repellent fraction of the lab chow infusion and was therefore used in repellency studies against several species of mosquitoes. The following species of mosquitoes were used: Culiseta incidens (Thomson), Anopheles quadrimaculatus Say, and Ae. aegypti. All three species of mosquitoes were maintained according to the procedures of Gerberg (1970).

The bioassay procedure described previously in Section E-1 was followed for An. quadrimaculatus females. However, slight modifications were made for Ae. aegypti and Cs. incidens females. For oviposition by Ae. aegypti, a 2.5x10-cm strip of paper towel (Crown No. 711) was placed in the liquid along the inside margin of all glass stender dishes to facilitate substrate oviposition of eggs by this species. In Cs. incidens test, black cloth discs (3 cm diameter) were placed under all stender dishes because a dark background had been found to induce a greater degree of oviposition in this species.

In calculating the OAI values, the number of egg rafts was used to represent the number of oviposition in Culiseta females. In Aedes and Anopholes, the females were examined after oviposition to determine the number of females that had oviposited, and this number was correlated with the number of eggs laid in both the treatment and the standard.

Aqueous solutions of butyric acid were prepared according to the procedure previously described.

Results and Discussion. Figure 2 shows the ovipositional activity of butyric acid at various concentrations against five species of mosquitoes. Ae. aegypti females exhibited a significant negative response only at the highest concentration of $6x10^{-1}$%. An. quadrimaculatus females were significantly repelled by $6x10^{-2}$ and $6x10^{-3}$% concentrations of butyric acid. Cs. incidens females were significantly repelled by butyric acid at $6x10^{-1}$% but significantly attracted at $6x10^{-3}$ and $6x10^{-4}$%. It was noteworthy that butyric acid showed remarkable properties of being both attractant and repellent for Cs. incidens females depending upon its concentration, inducing a repellent response at higher and an attractant response at lower concentrations.

From these studies, it is possible that the ovipositional behavior of various species of mosquitoes can be maneuvered by using different oviposition-modifying substances or different combinations of substances at various proportions and concentrations. It would be feasible that a particular formulation of oviposition-modifying substances could be designed for manipulating the ovipositional behavior of a given species of mosquito.

E-3. STRUCTURE-ACTIVITY RELATIONSHIP OF OVIPOSITION REPELLENTS

We previously found that acetic, propionic, isobutyric, butyric, isovaleric, and caproic acids, isolated from the lab chow infusions, induced negative oviposition responses in Cx. p. quinquefasciatus and Cx. tarsalis (Hwang et al. 1979). These carboxylic acids are homologous to one another and possess similar

biological activity against gravid female mosquitoes. To study the structure-activity relationship and to possibly procure more active compounds, some other aliphatic carboxylic acids and esters have been investigated for their ovipositional activity against Cx. p. quinquefasciatus.

Methods and materials. All aliphatic carboxylic acids and esters used in the study were of reagent grade. Because of their low solubility in water, the carboxylic acids and esters were processed in two ways for the bioassays. First, a carboxylic acid or an ester was dissolved in acetone, and an aliquot of the acetone solution was added to water to form an aqueous suspension with the specified concentration of the acid. The amount of acetone used was no more than 1% of water. A portion (4 ml) of the aqueous suspension of the acid was placed in a stender dish, which was then used together with a control dish in a bioassay unit for evaluation. Secondly, an acetone solution of an acid with known concentration was pipetted onto a paper disc (Whatman No. 5 filter paper, 7 mm diameter), and acetone was allowed to evaporate. The disc, which was impregnated with the carboxylic acid, was then placed on the surface of distilled water (4 ml) in a stender dish and subjected to bioassay. The former was designated as direct method, and the latter as disc method. The bioassay procedures described previously were followed for evaluating the aliphatic carboxylic acids and esters for their ovipositional activity.

Results and Discussion. Caprylic acid (octanoic acid) showed 100% repellency at the $6x10^{-3}$ and $6x10^{-4}$% concentrations against Cx. p. quinquefasciatus females, and therefore, was at least ten times more active than those lower aliphatic carboxylic acids previously identified from the lab chow infusions (Table 3). Capric acid (Decanoic acid) was significantly repellent at concentrations higher than $6x10^{-5}$% (0.6 ppm). This minimum effective concentation was 100 times lower than those of acetic and isobutyric acids. Maw (1970) reported that caproic acid showed repellency against Cx. restuans Theobald at 150-300 ppm. Artificial pools treated with caproic acid were initially repellent to mosquito oviposition, then gradually lost their repellency, became exceptionally attractive for oviposition, and finally became no more acceptable for mosquito oviposition than untreated pools. No quantitative work was attempted for determining the concentration effects of the acid.

Lauric acid (dodecanoic acid) was significantly active at $6x10^{-5}$% in the direct method and at $6x10^{-4}$% in the disc method, but myristic acid (tetradecanoic acid) was inactive at the concentrations tested.

Some of the higher carboxylic acids thus far studied exhibited much higher levels of repellency than the lower carboxylic acid previously identified. The present studies are still under way, and several other carboxylic acids including both odd- and even-numbered are under investigation for their ovipositional activity.

Although Perry and Fay (1967) found that some esters of lower carboxylic acids showed oviposition-modifying activity, we found that ethyl acetate, methyl propionate, ethyl propionate, methyl butyrate, and ethyl butyrate did not show any activity in our bioassay units at the $6x10^{-1}$ to $6x10^{-4}$% concentrations against Cx. p. quinquefasciatus (Figure 3).

E-4. LARVICIDAL ACTIVITY OF OVIPOSITION REPELLENTS

During the course of studying ovipositional activity of lower aliphatic carboxylic acids, we found that, at the $6x10^{-2}$% concentration, butyric acid caused 100% mortality in first-instar larvae of Cx. p. quinquefasciatus. We thus postulated that gravid female mosquitoes might have acquired, through evolutionary adaptation, the ability to avoid ovipositing in unsuitable sites in which toxic substances prevailed and therefore might be detrimental to the survival and development of their offspring.

Aliphatic carboxylic acids homologous to those identified as mosquito oviposition repellents were known to possess toxic effects on mosquito larvae at high dosages. Thus, Maw and House (1971) found caproic acid to be a good larvicide at 150 and 300 ppm. Quraishi (1972) reported some alkanoic and 2-alkenoic acids from C_6 to C_{12} to show good larvicidal activity against Ae. aegypti at 500 ppm. No attempts by these workers were made to determine the lethal dosage values.

In conducting field tests for assessing the efficacy of the oviposition repellents influencing the ovipositional behavior of mosquitoes, the measurement of larval populations is more tenable to sampling than that of ovipopulation. It is very difficult, if not impossible, to assess the potency of such larvicidal repellents which may cause larval mortality during bioassay tests and thus affect the accuracy of our evaluations. Prior to conducting field bioassays, the larvicidal studies of the existing and the potential repellents are, therefore, of prime importance. The larvicidal activity of some aliphatic carboxylic acids has been investigated against both young and mature larvae.

Methods and Materials. Twenty second- or fourth-instar larvae of Cx. p. quinquefasciatus were placed in 200-ml water in a 11-cm diameter polyurethene dish. The larval dishes were placed in a room kept at 27±1°C and under 14-hour photoperiod.

The test compounds were dissolved in acetone and serially diluted. No more than 1 ml of these solutions was added to the test dishes. Checks were treated with equal volumes of acetone only. The tests were run in three replicates and continued until adult emergence. Mortalities were read every 2-3 days.

All aliphatic carboxylic acids used for the bioassays were of reagent grade.

Results and Discussion. In preliminary tests, caprylic, capric, and lauric acids have been tested for larvicidal activity against second instars of Cx. p. quinquefasciatus at 1, 5, and 10 ppm. Although the studies are not complete as yet, these carboxylic acids have not shown considerable toxicity against mosquito larvae at these concentrations which are likely to be used in field studies.

As discussed in the preceding section, capric acid shows significant repellency against gravid female mosquitoes at 0.6 ppm, a concentration which will show no toxic effect against mosquito larvae. In our planned field

studies of assessing the role of repellents in manipulating mosquito oviposition behavior, capric and lauric acids are probably excellent candidate compounds for this purpose. Furthermore, they do not have the foul odor as do the lower acids.

E-5. SENSORY PHYSIOLOGY OF OVIPOSITION REPELLENTS

The antennae are probably the most important external sensory organs. Antennae extirpation in Ae. aegypti females caused a definite shift from a positive olfactory response to fatty acid esters to a lack of chemical orientation (Perry and Fay 1967). Extirpation studies conducted by Ikeshoji (1966b) with Cx. fatigans Wiedemann females indicated that the antennae were important in detecting substances responsible for the stimulative factor of breeding waters.

In Cs. inornata (Williston), the two labellar lobes at the tips of the proboscis bore hairs which were proven to be chemosensory in nature (Feir et al. 1961). Chemosensory hairs were identified on mosquito tarsi which were known to mediate responses to sugar and salt solutions (Frings and Hamram 1950, Slifer 1962, Salama and Ata 1972).

The present work concerns itself with the studies on the identification of the location of chemoreceptors which mediate negative oviposition responses of Cx. p. quinquefasciatus females to butyric acid (Kramer and Mulla 1979b).

Methods and Materials. Gravid Cx. p. quinquefasciatus females were removed from holding cages in groups of ten each and lightly anesthesized with carbon dioxide. To determine the involvement of various organs in the discrimination of substances that elicit negative oviposition responses, the proboscis, all tarsal segments and/or a variable number of antennal segments were extirpated with fine surgical microscissors under a dissecting binocular microscope.

The flagellar segments of the antennae were numbered from 1-13 beginning with the proximal segment. Varying numbers of segments were removed from either/or both of the antennae. When a whole antenna was extirpated, flagellar segments 3-13 were removed. When one-half of an antenna was extirpated, flagellar segments 8-13 were removed. In experiments which involved the extirpation of portions of one antenna or the whole antenna, these cuts were always made on the right antenna. In the tarsal extirpation experiments, cuts were made just dorsal of the tibial-tarsal joints. The removal of the proboscis was made as close as possible to its base.

Both untreated and CO_2 treated females (with all body parts intact) were used in control oviposition choice tests to determine the effect of CO_2 on oviposition behavior. According to Roth (1948), continuous CO_2 anesthesia for as long as 60 minutes has no effect on the feeding behavior and recovery of Ae. aegypti females. All females which had their body part removed were allowed at least two hours to recover before transfer to the oviposition bioassay units.

The oviposition bioassay procedures were carried out according to those described in Section E-1.

Results and Discussion. In preliminary experiments using control and no test substance, we found that the number of egg rafts deposited by Cx. p. quinquefasciatus females with various body parts extirpated (antennae, proboscis, or all tarsal segments) was not significantly different from that by normal, unextirpated females. Carbon-dioxide treated females also laid as many egg rafts as those untreated.

The results of our studies are listed in Table 4. Neither treatment of carbon dioxide nor extirpation of proboscis or all tarsal segments had any significant effect on the extent of negative oviposition response of the extirpated mosquitoes to $6x10^{-2}$% butyric acid. Partial or total extirpation of one or both antennae caused the extirpated mosquitoes to cease responding to $6x10^{-2}$% butyric acid. We have therefore concluded that the chemoreceptors for the perception of butyric acid is located in the antennae which is the most important sensory organ in mediating the negative ovipositional responses of the mosquitoes to the repellents.

When exposed to $6x10^{-1}$% butyric acid (Table 5), the female mosquitoes whose various sensory organs were extirpated, were still capable of making significant negative responses to this higher concentration of butyric acid. Even removal of multiple receptor system including tarsi and both antennae did not prevent the mosquitoes from responding to the concentrated acid. The only conclusion which can be deduced from these studies is that, in addition to the antennal chemoreceptors, probably other mechanisms exist for the female mosquitoes to perceive the repellents.

E-6. ISOLATION AND IDENTIFICATION OF OVIPOSITION ATTRACTANTS

In his studies on capric acid as an oviposition stimulant for mosquitoes, Maw (1970) discovered that capric acid which was initially repellent became attractive due to the presence of bacteria of the family Pseudomadaceae. He found that "bacteria responsible for the attractiveness were present in most of the pools and capric acid acted as a 'fertilizer', supplying the carbon needed for their rapid growth." Water taken from his attractive pools was found attractive to Cx. restuans, Ae. aegypti, Cx. pipiens L., and Cx. tarsalis.

Ikeshoji et al. (1975) confirmed Maw's findings and reported that Pseudomonas bacteria produces oviposition attractants for Ae. aegypti and Cx. p. molestus Forskal on capric acid and pelargonic acid (nonanoic acid) substrates. These workers speculated that the attractants would be the intermediate metabolites of these carboxylic acids.

No attempts were made by these researchers for the isolation and identification of the oviposition attractants, and whether capric acid acts as a "fertilizer" in the production of the attractants or whether the attractants are the intermediate metabolites of capric and pelargonic acids remain to be clarified further.

Concurrent with the oviposition repellent studies, techniques and procedures are being developed for the isolation and identification of the mosquito oviposition repellents produced in the chicken manure infusions.

In the initial stages, efforts are being made to separate active fractions from inactive materials and to isolate the oviposition attractants in semi-pure or pure forms for chemical identification.

Methods and Materials. The bioassay techniques described in Section E-1 were used for monitoring the activity of the infusions and fractions obtained in the isolation procedures. To determine the effect of dilution, these materials were also diluted with distilled water and bioassayed.

Chicken manure was collected from chicken ranches in southern California. dried in the air, and stored in plastic bags at room temperature. Infusions were prepared by adding tap water to 100 g dried chicken manure in a 10-liter glass # container until a 1% suspension was made. The resulting infusions were aged at room temperature. Aliquots were taken from the infusions every 2-3 days and bioassayed for their attractancy.

After about 9 days, the activity of the infusions reached the maximum. To separate volatile active compounds from nonvolatile inert materials, the infusion (8 liters) was steam-distilled under atmospheric pressure to give a distillate (16 liters) and a residue. The residue was diluted with distilled water to 8 liters, and an aliquot of the reconstituted residue was bioassayed. The distillate was also bioassayed.

The distillate was extracted three times with ether, and the ether layers were combined and dried over Na_2SO_4 or $MgSO_4$. The dried ether solution was evaporated to dryness to give a yellowish ether extract (0.3g). A portion of the ether extract (3.75 mg) was dissolved in acetone (1 ml), and the acetone solution was diluted with distilled water to 100 ml. The resultant aqueous mixture of the ether extract and its dilutions were subjected to bioassays.

Results and Discussion. Table 6 shows the ovipositional activity of the chicken manure infusions at different aging stages and their dilutions. The freshly prepared infusion was not active, but it began to show attractancy after two days of aging upon 1:1 dilution with water. The attractancy steadily increased thereafter, and in the ninth day of aging it reached an OAI of 0.92. At this point, the infusion was harvested for the isolation of the oviposition attractants.

Upon distillation, the active infusion yielded a distillate which showed some but insignificant attractancy (Table 7). The 1:3 and 1:7 diluted distillates, however, exhibited significant activity. The distillation residue did not show any significant attractancy except at the 1:1 dilution. The experiment results showed that the attractants were volatile and distillable with steam and that the ovipositional activity was dependent upon concentration of the attractants in water.

The distillate was extracted with ether to give an ether extract which upon reconstitution to the original concentration with water displayed significant ovipositional attractancy. The 1:1 dilution of the reconstituted aqueous mixture of the ether extract still elicited significant positive

response in Cx. p. quinquefasciatus females. Further dilution of the aqueous mixture caused disappearance of attractancy. Apparently, the oviposition attractants were soluble in organic solvents and therefore extractable with ether.

Although both distillate and ether extract were proven to be significantly attractive to ovipositing female mosquitoes, there was a decrease of activity in these two fractions as compared to the original infusion. Further investigation is needed for accounting for the decrease of attractancy during the process of isolation.

Our chemical and biological studies thus revealed that the ether extract contains active compounds with slightly declined but significant attractancy. Further separation and purification of the oviposition attractants is presently under way. Once purified active compounds are procured, identification work will be proceeded.

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Table 1. Relationship between acid concentration and the number of ovipositions (treatment plus standard) observed[a/]

Acid Concentration	Mean no. ovipositions/5 females[bc/] butyric *C. p. quinquefasciatus*	acetic *C. p. quinquefasciatus*	isobutyric *C. tarsalis*
6×10^{-6}	3.62 a	———	———
6×10^{-5}	3.37 ab	———	———
6×10^{-4}	3.12 abc	4.12 a	4.37 a
6×10^{-3}	2.62 bcd	4.00 a	3.87 ab
6×10^{-2}	2.37 cd	4.00 a	3.62 ab
6×10^{-1}	1.75 d	2.62 b	3.00 b

[a/] Results of tests with all other carboxylic acids tested against *C. p. quinquefasciatus* and *C. tarsalis* were found to be insignificant.

[b/] All values are means of 8 replicates.

[c/] Means followed by the same letter are not significantly different from one another at the 0.05 level of probability based on Duncan's multiple range test.

Table 2. Oviposition activity indices of combinations of lower carboxylic acids tested against gravid Culex mosquitoes (distilled water as a standard).

Total conc. of acids (%)	Test species and OAI (± S.E.)[a/]	
	C. p. quinquefasciatus	C. tarsalis
	Combination 1[b/]	
6×10^{-4}	-.13±.11	+.15±.05
6×10^{-3}	-.33±.47	-.30±.44
6×10^{-2}	-.75±.25**	-.80±.12**
6×10^{-1}	- 1 ± 0 **	- 1 ± 0 **
	Combination 2[c/]	
6×10^{-4}	-.23±.20	-.11±.15
6×10^{-3}	-.31±.21	-.53±.15**
6×10^{-2}	- 1 ± 0 **	-.83±.17**
6×10^{-1}	- 1 ± 0 **	- 1 ± 0 **

[a/] ** Significant from standard at $p < .01$.

[b/] The combination consisted of 3.79% acetic acid, 0.08% propionic acid, 1.43% isobutyric acid, 87.58% butyric acid, 3.12% isovaleric acid, and 3.40% caproic acid. All values are means of 4 replicates (5 ♀♀/replicate).

[c/] The combination consisted of equal proportions of acetic, propionic, isobutyric, butyric, isovaleric and caproic acids. All values are means of 8 replicates (5 ♀♀/ replicate).

Table 3. Oviposition activity indices of higher aliphatic carboxylic acids against Cx. p. quinquefasciatus.

Acid	Conc. (%)	OAI ± S.E. (Replicates)[a/] Disc. Method	Direct Method
Caprylic	6×10^{-4}	-1.00±0 (6)*	-1.00±0 (6)*
	6×10^{-3}		-1.00±0 (6)*
Capric	6×10^{-6}	-0.22±0.22 (6)	
	6×10^{-5}	-0.80±0.14 (6)*	-0.58±0.20 (6)*
	6×10^{-4}	-1.00±0 (6)*	-1.00±0 (6)*
	6×10^{-3}		-1.00±0 (6)*
	6×10^{-2}		-1.00±0 (6)*
Lauric	6×10^{-5}	-0.17±0.21 (6)	-0.56±0.21 (6)*
	6×10^{-4}	-0.72±0.13 (6)*	
	6×10^{-3}		-1.00±0 (6)*
Myristic	6×10^{-5}	+0.38±0.20 (6)	
	6×10^{-4}	-0.06±0.29 (6)	

[a/] * Significant from the standard at $p < 0.05$.

Table 4. Oviposition responses of C. p. quinquefasciatus females subjected to various extirpations to $6x10^{-2}$% butyric acid.

Organs extirpated	Oviposition responses[a/] OAI ± S.E.[b/]	$\bar{x}$ ovipositions/5 ♀♀
Intact	-.74±.10**	3.75
Intact (CO_2)	-.67±.10**	3.63
All tarsi	-.77±.11**	3.38
Proboscis	-.65±.11**	4.25
Both antennae	-.25±.15	3.13
One antenna	-.19±.17	4.37
½ both antennae	+.34±.22	4.37
½ one antenna	-.25±.27	3.00

[a/] All values are means of 8 replicates.

[b/] ** Significant from standard at $p < .01$.

Table 5. Oviposition responses of C. p. quinquefasciatus females subjected to various extirpations to $6x10^{-1}$% butyric acid.

Organs extirpated	Oviposition responses[a]	
	OAI ± S.E.[b]	$\bar{x}$ ovipositions/5 ♀♀
Intact	-1±0**	2.75
Intact (CO_2)	-1±0**	2.62
All tarsi	-.92±.08**	2.13
Both antennae	-1±0**	3.13
One antenna	-1±0**	2.88
All tarsi and antennae	-1±0**	1.63

[a] All values are means of 8 replicates.

[b] ** Significant from standard at $p < .01$.

Table 6. Oviposition activity indices of aging chicken manure infusions against Cx. p. quinquefasciatus.

Infusion Age (Days)	Dilution[a/]	OAI±S.E. (Replicates)[b/]
0	None	-0.45±0.06
0	1:1	-0.03±0.13
2	None	+0.33±0.13
2	1:1	+0.60±0.15**
6	None	+0.43±0.08*
6	1:1	+0.67±0.22**
6	1:3	+0.57±0.13**
7	None	+0.57±0.22**
7	1:1	+0.36±0.17
7	1:3	+0.45±0.08*
7	1:7	+0.80±0.09**
9	None	+0.78±0.09**
9	1:1	+0.72±0.14**
9	1:3	+0.87±0.08**
9	1:7	+0.92±0.08**

[a/] Infusion:distilled water.

[b/] *Significant from the standard at $p < 0.05$.

**Significant from the standard at $p < 0.01$.

Table 7. Oviposition activity indices of fractions obtained from chicken manure infusion against Cx. p. quinquefasciatus.

Fraction	Dilution[a/]	OAI±S.E. (replicates)[a/]
Distillate	None	+0.28±0.20
Distillate	1:1	+0.24±0.28
Distillate	1:3	+0.42±0.15*
Distillate	1:7	+0.70±0.09**
Residue	None	+0.21±0.14
Residue	1:1	+0.38±0.27*
Residue	1:3	+0.22±0.15
Residue	1:7	+0.05±0.15
Ether extract[c/]	None	+0.49±0.23*
Ether extract	1:1	+0.47±0.20*
Ether extract	1:3	+0.28±0.17
Ether extract	1:7	+0.23±0.24

[a/] Infusion:distilled water.

[b/] *Significant from the standard at $p < 0.05$.
**Significant from the standard at $p < 0.01$.

[c/] Reconstituted by dissolving 3.75 mg of the ether extract in 1 ml of acetone and diluting the acetone solution with distilled water to 100 ml.

Figures

FIGURE 1 - Oviposition Activity Indices (OAI) of carboxylic acids tested against C. p. quinquefasciatus and C. tarsalis females. All values are means of 8 replicates (5 ♀♀/replicate) ± S.E.

FIGURE 2 - OAI of butyric acid tested against different mosquito species. All values are means of 8 replicates ± S.E.

FIGURE 3 - OAI of carboxylic acid esters tested against C. p. quinquefasciatus females. All values are means of 6 replicates ± S.E.

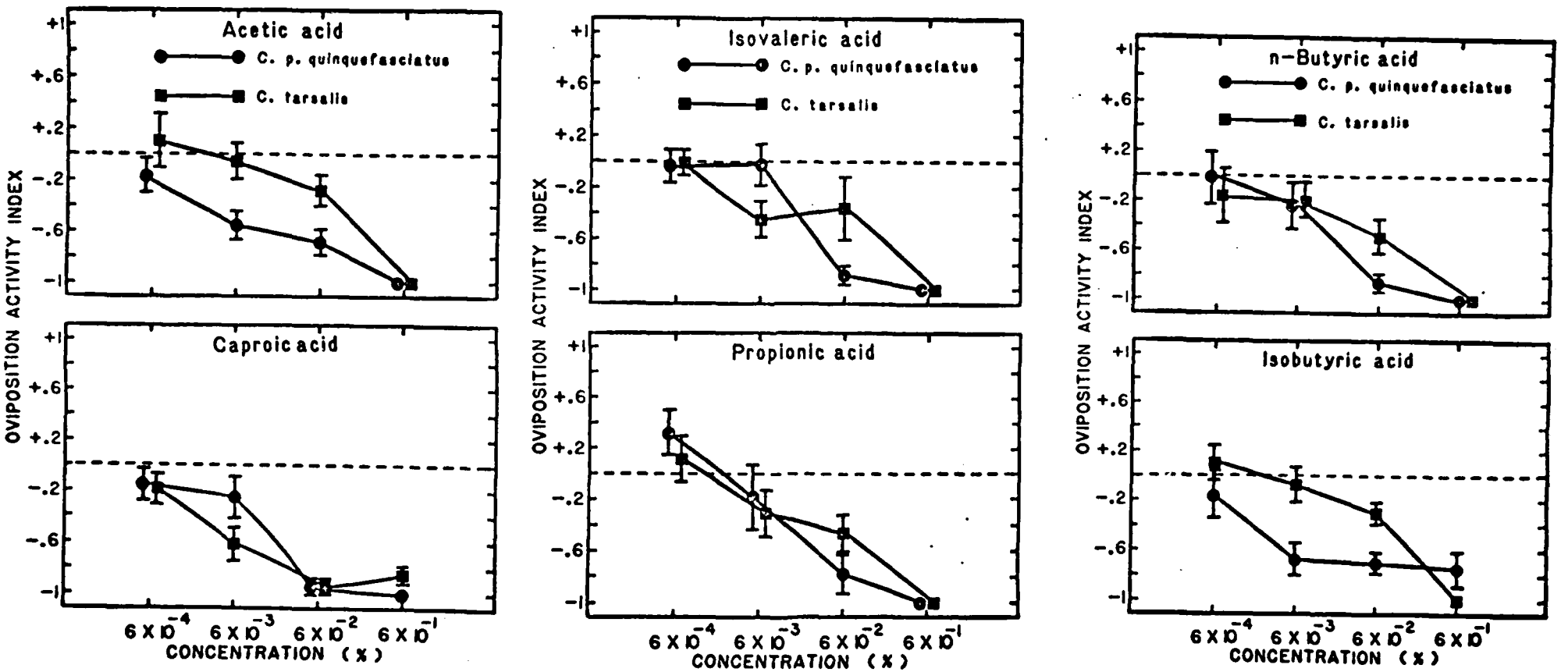
Acetic acid
C. p. quinquefasciatus
C. tarsalis
Caproic acid
Isovaleric acid
Propionic acid
n-Butyric acid
Isobutyric acid
OVIPOSITION ACTIVITY INDEX
+1
+.6
+.2
-.2
-.6
-1
6 X 10^-4
6 X 10^-3
6 X 10^-2
6 X 10^-1
CONCENTRATION (%)

Figure
1

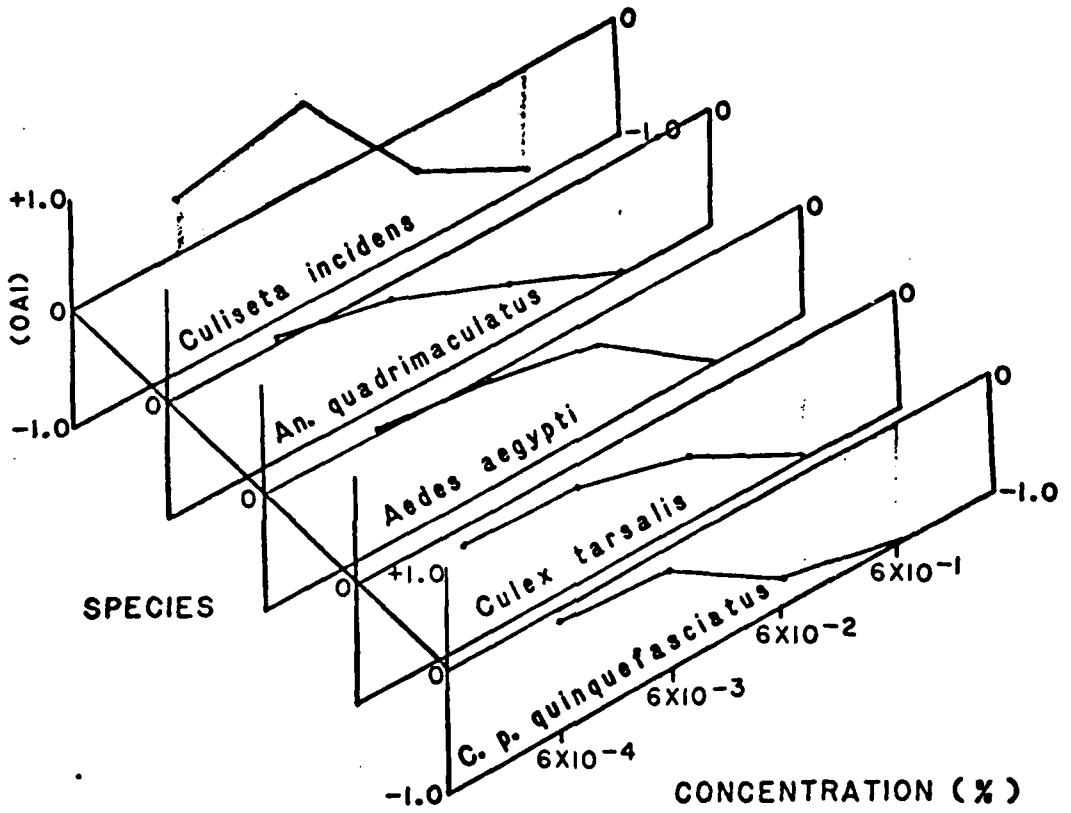
+1.0
0
-1.0
(OAI)
Culiseta incidens
An. quadrimaculatus
Aedes aegypti
Culex tarsalis
C. p. quinquefasciatus
SPECIES
6X10-1
6X10-2
6X10-3
6X10-4
CONCENTRATION (%)

Figure 2

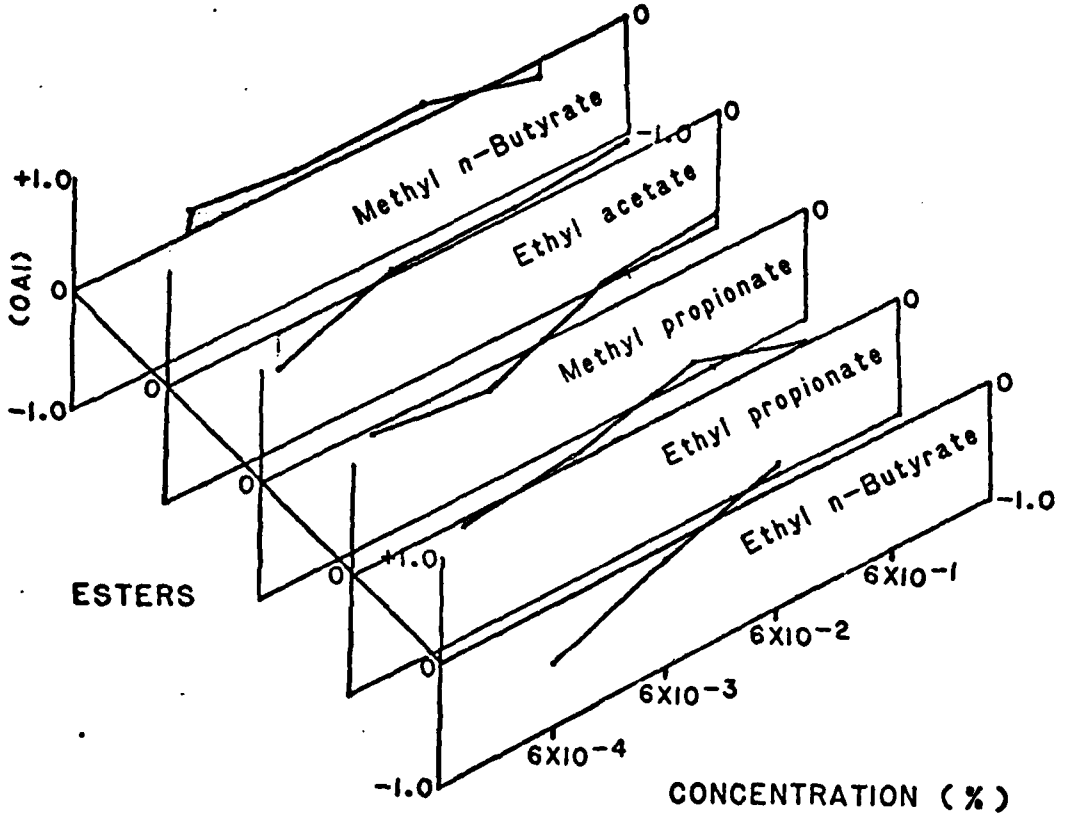
+1.0
0
-1.0
(OAI)
Methyl n-Butyrate
Ethyl acetate
Methyl propionate
Ethyl propionate
Ethyl n-Butyrate
ESTERS
6X10-1
6X10-2
6X10-3
6X10-4
CONCENTRATION (%)

Figure 3

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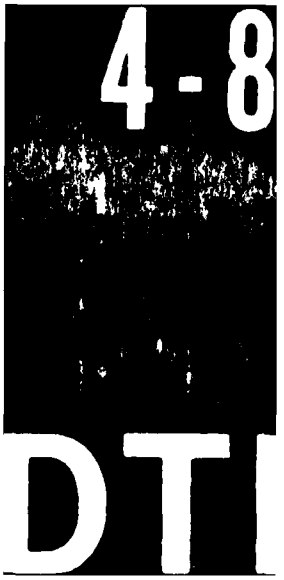
4-8
DTI